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Original Article

Optimization modeling of single-chain antibody against hepatoma based on similarity algorithm

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Abstract: The purposes was to establish optimal modeling of single-chain antibody molecules based on similarity algorithm and seek the connecting peptides that had the minimal effect on the structure and bioactivity of the variable region of heavy chain (VH) and that of light chain (VL) in a single-chain antibody against liver cancer. After the Linker with different lengths (n=0~7) had been added into single chain fragment variable (ScFv), modeling of the overall sequences of VH, VL and ScFv were conducted respectively. Meanwhile, the peptide chain structure of $(Gly_4Ser)_n$ was adopted for the connecting peptide. Then the spatial spherical shell layer alignment algorithm based on spherical polar coordinates was utilized for comparing the structural similarity of VH and VL before and after adding connecting peptide. Equally, in order to determine the stability of VH and VL, MATLAB was applied for analysis of the fore and aft distances and the diffusion radius. Indirect ELISA method was used to detect single-chain antibody immunological activity of Linker with different lengths. The MTT assay was utilized for the examination of the inhibition rate of single-chain antibody with different lengths of Linker to liver cancer cell. When n=4, the structural similarity between VH together with VL and their original ones was the highest. When n=3, the influence of connecting peptide on the stability of VH and VL was minimum. When n>3, the fore and aft distances changed little due to the increase and fold of the length of peptide chain. The results of ELISA detection showed that when n=4, affinity of single chain antibody to liver cancer cells was much higher. The MTT test also indicated that when n=4, the inhibition rate of the connecting peptide on hepatoma carcinoma cell reached the highest, and that came second when n=3. When n=4, the structural stability and biological functions of anti-hepatoma single-chain antibody were both favorable. This study has provided a basis for the design and construction of single-chain antibody.

Keywords: HscFv4-16, connecting peptide, similarity algorithm, optimization, modeling

Introduction

Primary liver cancer is one of the most common digestive system malignancies in clinic of which up to 90% are hepatocellular carcinoma (HCC). In the European and American countries, the incidence of liver cancer is the fifth among malignant tumors. The incidence is much higher in China, Southeast Asia and other regions, with its mortality being consistently in the second place among malignancies, even for the first place [1, 2]. It has seriously threatened human being's life and health, and the incidence and mortality of liver cancer are still on the rise around the world [3]. Currently, the main treatments of liver cancer are surgical excision, systemic chemotherapy and biological targeted therapy. However, most of the liver cancers are primary tumors and the pathogenesis is occult. The majority of which to be found belongs to the advanced stage, losing the best opportunity for operation. The chemotherapy drug has obvious side effects due to lack of specificity of liver cancer cells. Therefore, it is extremely urgent to make an insight into the molecular mechanism of the occurrence and development of liver cancer and find out the diagnostic target.

In recent years, the intensive study on the antibodies of liver cancer has brought a new prospect to the diagnosis and treatment of liver cancer. ScFv exhibits the advantages of small molecules, strong penetrating power, fast clearance, low heterology and easy to mass production [4] et al. It has the ability to neutralize the viruses and toxins and act as a guide carrier for intracellular immunization. It shows great potential in the field of diagnosis and treatment of liver disease. Studies indicated that HscFv4-

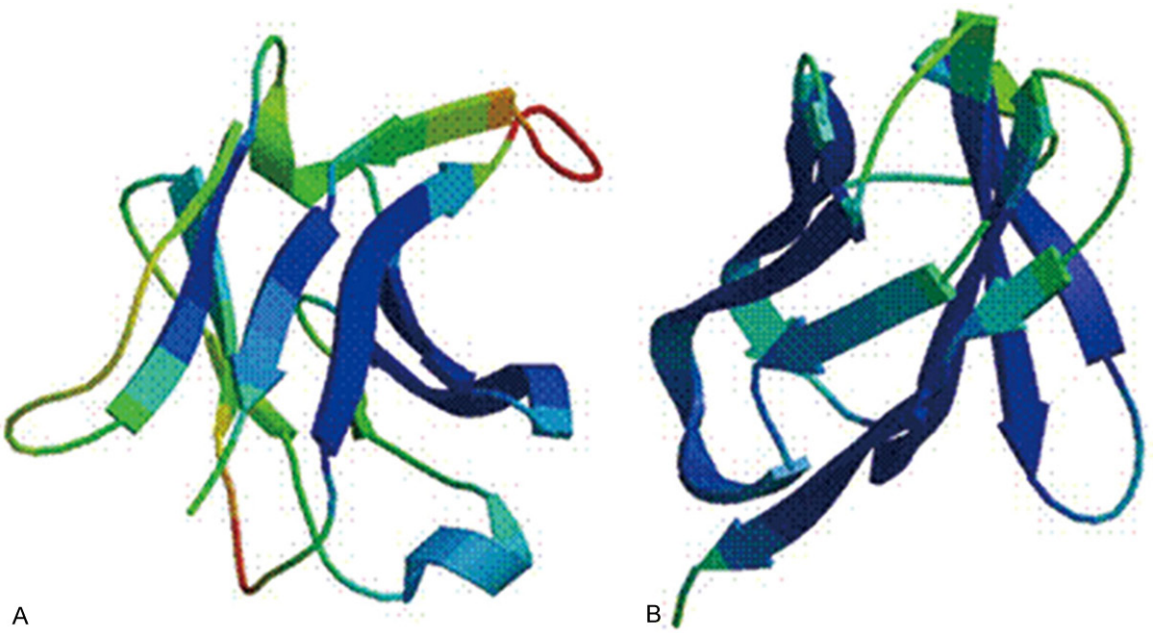

Figure 1. The three-dimensional structures of VH and VL of ScFv (A: VH; B: VL).

16 possessed the typical features of ScFv and can be integrated with the liver cancer cells and the antigenic specificity in liver cancer tissues. Therefore, it is feasible to treat HscFv4-16 as the single-chain antibody in the speci ficity of liver cancer cells [5]. ScFv is a recombination protein connected by VH and VL through a section of connecting peptide. Many studies have learned that a single protein was formed by two derived factors gene of fusion proteins. After the detection of anti-tumor activity, it was found that the fusion protein had a dual activity. Compared with the function and activity of the derived factors, however, the affinity and antitumor activity of fusion protein had changed [6-9], which may attribute to changes in the structure of fusion protein. The main factors in determining the biological activity of fusion protein may include: (1) the spatial position of derived factors that depends upon the activity of functional domain of the derived factor protein; (2) the receptor structures of the derived factors and the relationship between them; (3) size and complexity of the intermediate joint, and other factors that may affect the spatial structure of the protein. At present, there were few reports about the effect of interchain connecting peptide on the bioactivity of single-chain antibody. Consequently, it will be of great significance in the screening of the connecting peptide and the development of single chain antibody to lucubrate the impact of the connecting peptide to the biological activities of single chain antibody. This study tried to investigate the optimum length of the linker peptide that had no effect on the biological activities of anti-hepatoma single-chain antibody. It not only built a foundation for the design of the interchain connecting peptide and the construction of single chain antibody, but offered certain theoretical basis for targeted biotherapy of liver cancer.

Materials and methods

The experiment was done in accordance with the relevant ethical and legal standards established and accepted by our host institution or in accordance with the Helsinki Declaration of 1975. The informed consent has been obtained.

Materials

The amino acid sequence of HscFv4-16 (specific single chain antibody of hepatocellular carcinoma cell) derived from NCBI (GenBank: DQ640759.1; Bethesda, MD, USA). The amino acid sequence is:

MAQVKLQQEGTEVVKPGASVKLSCKASGYIFTS-YDIDWVRQTPEQGLEWIGWIFPGEGSTEYNE-KFKGRATLSVDKSSSTAYMELTRLTSEDSAV-YFCARGDYYRRYFDLWGQGTTVTVSS (GGGGSG-GGGSGGGGS) DIELTQSPAIMSASPGERVTMTCS-ASSSIRYIYWYQQKPGSSPRLLIYDTSNVAP-GVPFRFSGSGSGTSYSLTINRMEAEDAATYYCQ-EWSGYPYTFGGGTKLELKR.

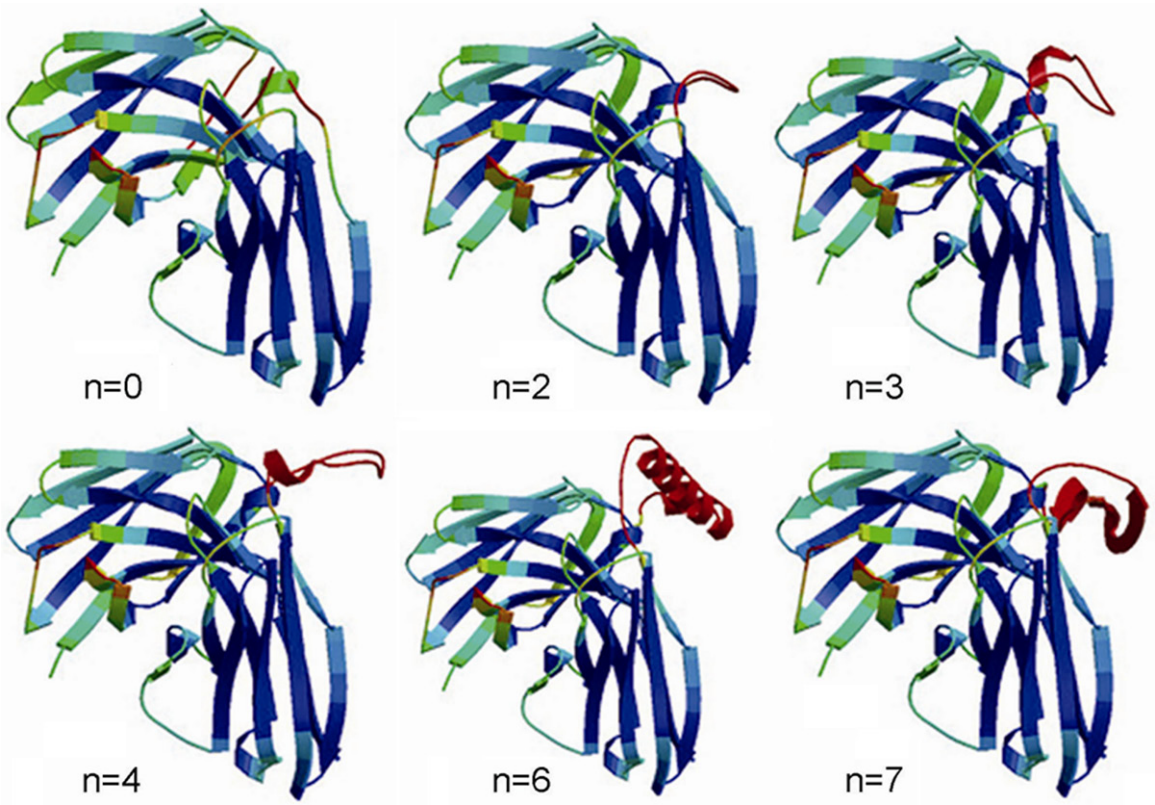

Figure 2. The three-dimensional structures of protein molecules when n=0, 2, 3, 4, 6, 7.

Table 1. The results of similarity comparison

Layer	2	3	4	Mean value	Layer	2	3	4	Mean value	Total mean value
VH0	0.9551	0.8235	0.5066	0.76173	VL0	0.9824	0.9356	0.8887	0.9356	0.8487
VH1	0.9536	0.8286	0.4987	0.76030	VL1	0.9997	0.9407	0.8863	0.9422	0.8513
VH2	0.9632	0.8096	0.5209	0.76457	VL2	0.9985	0.9458	0.8904	0.9449	0.8547
VH3	0.9625	0.8096	0.522	0.76470	VL3	0.998	0.944	0.8832	0.9417	0.8532
VH4	0.9625	0.8144	0.522	0.76630	VL4	0.9982	0.9458	0.8904	0.9448	0.8556
VH5	0.9632	0.8096	0.522	0.76493	VL5	0.9981	0.9421	0.8835	0.9412	0.8531
VH6	0.9627	0.8096	0.522	0.76477	VL6	0.9951	0.9437	0.8858	0.9415	0.8532
VH7	0.9632	0.8096	0.522	0.76493	VL7	0.9981	0.9421	0.8835	0.9412	0.8531

Liver cancer cell lines HepG2 and Bel-7402 were saved in our lab. We ourselves constructed the common expression vector of single-chain antibody with different lengths of linker and purified its expression.

Methods

Three-level modeling SWISS-MODEL (Basel, Switzerland) approach was used for homology modeling of HscFv4-16. Then modeling of the sequence of VH and VL were conducted respectively. This aimed to obtain the corresponding three-dimensional spatial structure and pdb file (pdb code: VH: 4izx; VL: 1baf), which could be regarded as the reference data.

Selection of the connecting peptide

Currently, the most widely used linker is $(Gly_4Ser)_3$ with a rigid structure. It was designed by Huston et al, according to the antibody variable region structures from X-ray crystal diffraction analysis and the result of computer-aided analysis. That is, 15 amino acid residues include four glycines and one serine repeated three times [10, 11]. This linker not only connects the N-terminal with the C-terminal of V

Table 2. Fore and aft distances and radius of VH, VL and VHL before and after adding the Linker

	Fore and aft distance	Radius		Fore and aft distance	Radius	Fore and aft distance of Linker
VH0	33.5855	26.9256	VL0	28.9176	21.7122	—
VH1	35.5712	26.7185	VL1	26.3304	21.8663	9.0376
VH2	40.4282	26.5629	VL2	33.7021	21.7527	28.127
VH3	39.8876	26.5639	VL3	35.4337	21.9221	32.2359
VH4	39.3256	26.5843	VL4	34.4792	21.7548	33.7352
VH5	39.8852	26.5890	VL5	35.4055	21.7268	33.7295
VH6	39.8816	26.5982	VL6	35.4183	21.7353	32.4324
VH7	39.8860	26.5911	VL7	35.3986	21.7281	33.0345
VH	25.2156	26.5451	VL	37.6749	27.7985	—

region, at the same time, it also can tighten the VH and VL without affecting the interaction between them [12]. Among the amino acid, glycine is the smallest in molecular weight and with the shortest side chain, which can increase the flexibility of the side chain. Serine is a sort of hydrophilic amino acid and can increase the hydrophilicity of the linker [13]. Hence, (Gly_4Ser) was selected in this paper as the basic structural unit of the single-chain antibody connecting peptide.

Modeling

To obtain different amino acid sequences, this paper tried changing the length of the connecting peptide, i.e., changing of repeating unit n of Gly_4Ser. In general, the connecting peptide of ScFv is of 5 to 40 amino acids in length. The longer the connecting peptide is, the less stable of ScFv structure is. In the modeling process, when n=8 (i.e., the length of connecting peptide is 40 amino acids), the homology of the template, which has been searched, was too low. The modeling declared a failure. Thus, in this study the length of the connecting peptide is n=0~7. The three-level structure of single chain antibody of the connecting peptide with different length and data file of pdb structure were obtained by SWISS-MODEL homology modeling method with an aim of getting the corresponding and overall three-dimensional data and VHn, VLn data.

Structure comparison and stability analysis

There are lots of ways in analyzing protein similarity by using the spatial location information of the molecule. It can mainly be divided into two categories. One is the geometric detail comparison based on amino acids α carbon atom skeleton of the protein peptide chain. The other is the correlation analysis according to the topology connection characteristics between the basic construction units. This article adopted the spatial shell hierarchical algorithm based on the spherical polar coordinates, proposed by Jianhua Zhang [14], to calculate the similarity. Firstly, the three-dimensional coordinates of the structural model listed in the pdb file were converted into the spherical polar coordinates using the formula (1, 2, 3):

$$r = \sqrt{x^2 + y^2 + z^2} \quad r \in [0, +\infty) \tag{1}$$

$$\varphi = \arctan\left(\frac{y}{x}\right) \quad \varphi \in [0, 2\pi) \tag{2}$$

$$\theta = \arccos\left(\frac{z}{r}\right) \quad \theta \in [0, \pi] \tag{3}$$

According to the radius in the spherical polar coordinate system, we divided the layer. The shape of each layer can be considered as a spherical shell with the origin of coordinate as the centre of sphere. Based on the hierarchy of protein molecules, the number of same atoms in different layers was counted and the summation of atom numbers was calculated. The vectors were substituted into function (4), and the similarity of targeted atom can be obtained. The atom numbers in enclosed layers were stored in the multi-dimension ai and bi according to the order of hierarchical division. Then, the value of each atom was performed weighted average. Finally, the similarity of the comparison protein molecules was obtained.

Secondly, the largest space occupied by molecules was divided

$$\cos(a, b) = \frac{\sum_{i=1}^{n} a_i b_i}{\sqrt{\sum_{i=1}^{n} a_i^2 \sum_{i=1}^{n} a_i^2}} \tag{4}$$

The stability of ScFv can be represented by the fore and aft distance or the diffusion radius. The fore and aft distance refers to the distance

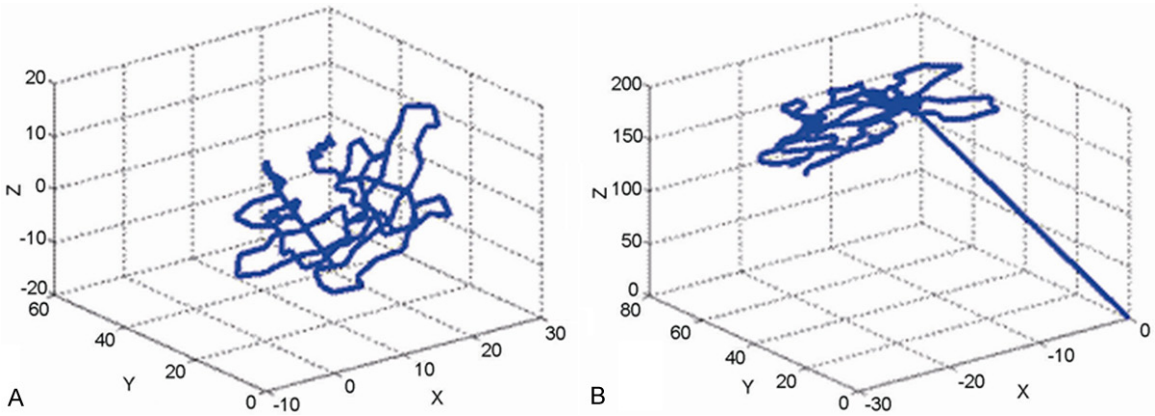

Figure 3. α-carbon atoms paths of the original VH and VL (A: VH; B: VL).

between first α-carbon atom and the last α-carbon atom in the protein. MATLAB (R2012a, The MathWorks, Inc., Natick, MA, USA) was used to calculate the fore and aft distance of the original structure of the VH and VL and the fore and aft length and maximum diffusion radius of VHn and VLn when added connecting peptide with different length (n=0~7).

Gene identification of single-chain antibody

Single-chain antibody expression vectors constructed by linker containing different length were identified using PCR (Bio-Rad Laboratories, Ltd., UK). PCR products were analyzed using 1.0% agarose gel electrophoresis (Bio-Rad Laboratories, Ltd., UK).

Detection of HscFv-16 immunological activity using ELISA method

Hepatocellular cancer cells HepG2 and Bel-7402 in the logarithmic phase were collected and inoculated in 96-well plates (Shanghai Chuanxiang Biotechnology Co., Ltd., Shanghai, China) of 5 × 10^4 cell/mL, 100 μL/well. It was cultured for 24 h at 37°C, washed 3 times with PBS (Sigma-Aldrich Co. LLC., St. Louis, MO, USA), and immobilized by 2.5% glutaraldehyde (Sigma-Aldrich Co. LLC., St. Louis, MO, USA) for 5 min at 37°C. After washing, equimolar HscFv4-16 connecting linker with different length was added to each well as the primary antibody. PBS was added in negative control well and incubated for 2 h at 37°C. 100 μL Goat-anti-Mouse HRP antibody (Sunshine Biotechnology Co., Ltd., Nanjing, China) was added to each well after washing, and also incubated for 2 h at 37°C. Substrate TMB (Bio-Rad Laboratories, Ltd., UK) was added after washed with PBS, then coloration at 37°C away from light for 5 min. Then 100 μL stop buffer (1 mol/L H_2SO_4) was added to each well. Microplate reader (Model 680; Bio-Rad Laboratories, Ltd., UK) was used to detect the OD value at a wavelength under 450 nm.

Examination of the inhibition ratio of HscFv4-16 on the hepatocarcinoma cell by MTT assay

The hepatoma cell lines HepG2 and Bel-7402 in logarithmic phase were selected and adjusted to (2~4) × 10^5 cell/ mL. Then they (100 μL/well) were inoculated in a 96-well culture plate. Each well was added 100 μL HscFv4-16 containing different length of connecting peptides and 3 parallel holes were made in each connecting peptide. One group of carcinoma cell suspension without antibody was set as control group, another group with culture solution as zero hole. Afterwards, 100 μL MTT (0.2 mg/mL) was added into each well and incubated at 37°C for 4 hrs. The supernatant was then removed and 200 μL alkalizing DMSO (10% glycine-NaOH buffer solution, Sigma-Aldrich Co. LLC. St. Louis, MO, USA) was added. After incubation for 1 h, the wells were oscillated and *A* value was determined at 570 nm by ELIASA. Each well was measured repeatedly more than three times. The results were basically the same and the median test values were selected as the standard. The activity level of HscFv4-16 was expressed by the inhibition rate of hepatoma cell and the formula is as follows: Inhibition rate=1-*A* value of experimental group/*A* value of control group × 100%.

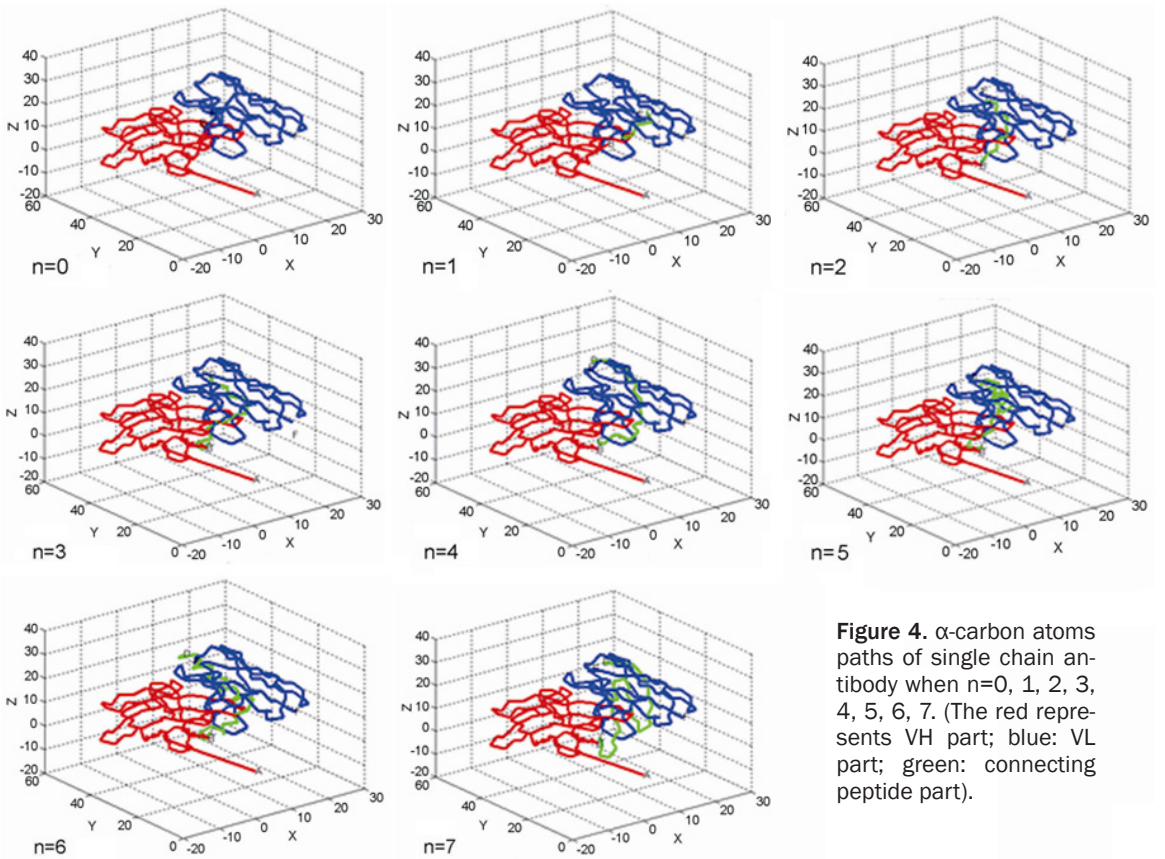

Figure 4. α-carbon atoms paths of single chain antibody when n=0, 1, 2, 3, 4, 5, 6, 7. (The red represents VH part; blue: VL part; green: connecting peptide part).

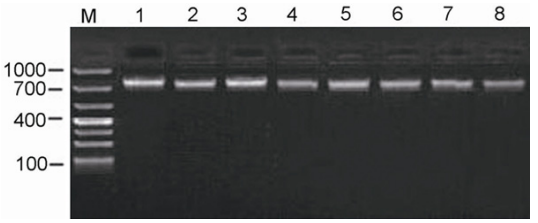

Figure 5. PCR identification of single chain antibody (1~8 represent the electrophoresis results of single chain antibody when n=0~7).

Results

Original three-dimensional structure of VH and VL in ScFv

Homology modeling method was used to model the original structure of the VH and VL of ScFv. The results were illustrated in **Figure 1**.

The three-dimensional structure of connecting peptide with different lengths (n=0~7) in ScFv

After homology modeling to ScFv of connecting peptide with different length (n=0~7), we displayed part of the modeling results (**Figure 2**).

As can be seen in **Figure 2**, the three dimensional structure of protein molecules was obviously different when n took different values. In addition, the single structure of VH and VL differed from the combined structure after adding n Gly_4Ser. The length of connecting peptide hence directly affected the spatial structure of the protein.

Similarity and stability analysis of VH and VL before and after adding the connecting peptide

After the homology modeling to the three dimensional structure of ScFv, the protein similarity algorithm based on the spherical polar coordinates was used for comparison of the similarity of VH and VL before and after adding the connecting peptide. After numerous trials by applying this algorithm, it was indicated that the similarity was more accurate and stable when the protein was divided into three layers. To ensure the accuracy of the results, the protein was divided into two, three and four layers respectively. Then the average value of three similarities was figured out as the final result. The higher similarity showed that the impact of

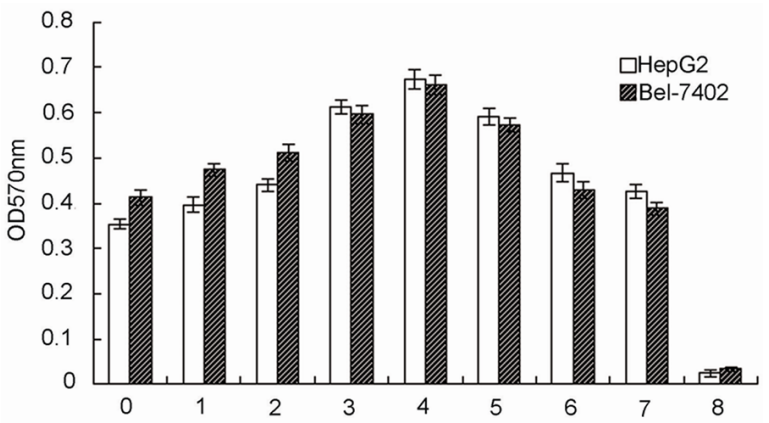

Figure 6. Detection of HscFv4-16 immunological activity by ELISA method. (0~7 represent HscFv4-16 with different length of Linker when n=0~7. 8 is the blank control).

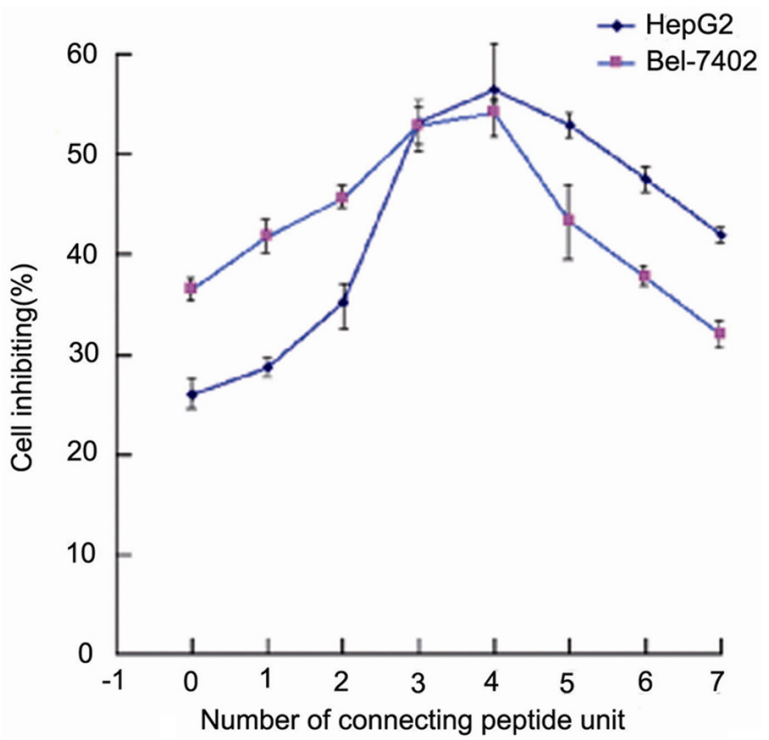

Figure 7. Inhibitory rate of HscFv4-16 on the liver cancer cells.

Linker peptide to the protein structure and the activity of the single chain antibody were both minimal. **Table 1** showed that the similarity between VH and original VH was the highest when n=4. The similarity between VL and original VL was the highest when n=2. After comprehensive analysis, it was found when n=4, the overall similarity of single chain antibody was the best before and after adding connecting peptide. While n took other different values, the overall similarity showed no significant difference.

Table 2 displayed the results of VH, VL, end-to-end distance of Linker and the radius of the spherical polar model before and after adding the connecting peptide. It showed that the diffusion radius of VH was most close to the original radius when n=2. When n=3, diffusion radius of VL was the closet to the original radius. In general, whatever values n took, the diffusion radius were of little difference. In addition, when n=3, the end-to-end distance of the VL is closest to the original range of VL. When n≤3, the end-to-end distance of the linker peptide increased as the peptide chain became longer. In contrast, when n>3, due to the increase and fold in the peptide chain, the end-to-end distance of the linker peptide changed a little. It proved that when n=3, the linker had the minimum impact on the stability of VH and VL molecule.

The α-carbon atoms path structure of the original VH, VL and the single-chain antibody after adding the linker in the positions of the space coordinate system was built by Matlab2008. In **Figures 3**, **4**, changes of the fore and aft distance of VHn and VLn were more intuitively presented and compared. As can be seen, a small change occurred to the overall radius of the molecule after adding the peptide chain with different lengths.

Gene identification of single-chain antibody

The established expression vectors of single-chain antibodies with different lengths of Linkers were identified. After analyzing PCR products by agarose gel electrophoresis, relevant DNA fragment was obtained at about

730~850 bp (**Figure 5**). The sequencing results were entirely consistent with the expected sequence.

Detection of HscFv4-16 immunological activity by ELISA method

The affinity between HscFv4-16 with different length of linker and liver cancer cell HepG2 and Bel-7402 was detected using ELISA test (**Figure 6**). The results showed that, when n=4, the affinity was relatively high. When n>4, the affinity reduced slightly.

Detection of inhibition rate of HscFv4-16 on hepatoma cell by MTT assay

MTT method was applied to examine the inhibition rate of HscFv4-16 with different lengths of linker on hepatoma carcinoma cell HepG2 and Bel-7402. In **Figure 7**, it was indicated that the inhibition ratio of HscFv4-16 on hepatoma carcinoma cell HepG2 reached 56.47% when n=4, the highest level. When n=3, the inhibition rate was 53.2%. While that reached 26% when n=0, the lowest level. In terms of examination of the inhibition ratio on Bel-7402, it reached 54.15% when n=4, the highest level; while when n=3, the inhibition ratio was 52.9%. When n=7, it reached the lowest level, but both exceeded 26%. Generally, when n=4, the inhibition rate of HscFv4-16 on the liver cancer cell HepG2 and Bel-7402 was the highest.

Discussion

The molecular weight of single-chain antibody is only about one sixth of the complete antibody. It has more superior applications in clinic than the complete antibody, such as low immunogenicity, strong penetrability of solid tumors, easy transformation of the genetic engineering, thus it has become a guide in tumor diagnosis and treatment and has broad application prospects. The connecting peptide between the VH and VL of single-chain antibody should have appropriate length. The short connecting peptide will affect the free folding space of the protein, accordingly affecting the structure and function of the protein. If the peptide chain was too long, it will affect the stability of ScFv. At the same time, it may increase the immunogenicity of ScFv, resulting in the reaction against heterogonous protein [15, 16]. Therefore, screening the suitable length for Linker length is particularly important.

In recent years, comparative studies on the spatial structure of albuminoid found that the three-dimensional structure of the protein is more conservative than its primary sequence, and it is more reliable to predict the spatial conformation of the target protein by albuminoid [17]. In addition, the structure of antibody has been successfully predicted by a number of experiments [17, 18]. Zhang JH [19, 20] has successfully studied the function of protein by similarity algorithm based on spherical polar coordinates. Three-dimensional modeling of protein structure can help researchers understand the spatial structure and biological and physiological functions of the antibody, based on which they can reshape the antibody molecules. Undoubtedly, this has great significance in developing the antibody engineering.

The property and function of molecule are determined by its structure. The spatial structure of proteins determines their biological function. Similarity comparison of protein, therefore, should be conducted at the level of its structure [21, 22]. In this study, homology modeling and bioinformatics algorithms et al were used to compare the change of variable region structure in the single chain antibody when the connecting peptide changed in length. Furthermore, it analyzed the optimum connecting peptide length of the single chain antibody by comparing the similarity. The results illustrated that the similarity between VH and the original VH was the highest when n=4. The similarity between VL and the original VL was the highest when n=2. The overall similarity is the best when n=4, indicating that Scfv-4 was most close to the original structure. When n=3, it has the minimum difference to the original radius. What's more, when n=3, the fore and aft distance of VL is the closet to that of the original VL, which indicated that its effect on the molecular stability of VH and VL was the least and was easy to fold. The results are consistent with the study conducted by Huston [23, 24].

So far, many studies were regarding the impact of the length of linker peptide on the activity of ScFv. Gustavsson [25] proposed that the connecting peptide used in the construction of single chain bispecific antibodies was 4-44 amino acids in length. Le [7] et al put forward that the interchain length of the connection peptide was 6-27 amino acids.

A variety of studies have shown that if the connecting peptide is 15 amino acids, all aspects of the single chain antibody, such as physical and chemical properties, biological activity, expression level, are able to reach a favorable degree [26, 27]. This study first established a theoretical modeling for comparative analysis, and initially identified the optimal length of the connecting peptide. Then MATLAB tool was used to calculate the changes of the end to end distance and the diffusion radius. Afterwards, it analyzed changes of stability of each model structure. The results confirmed once again that the sequence of the interchain connecting peptide could affect the biological activity and stability of single-chain antibody. Meanwhile, the structure of the connecting peptide also has an important impact on the function of single-chain antibody. ELISA and MTT assay test indicated that when n=4, the single chain antibody against hepacellular carcinoma has the ideal structure and biological activity. When n=4, the affinity between HscFv4-16 and hepatoma carcinoma cells HepG2 and Bel-7402 was relatively high. The inhibition rate of HscFv4-16 on HepG2 and Bel-7402 reached 56.47% and 54.15%, respectively. In summary, when n=4 (connecting peptide is 20 amino acids), the structure and bioactivity of single chain antibody anti-hepatoma are favorable, and it can be used as the connecting peptide in constructing anti-hepatoma single chain antibody. This study provided a new way of thinking in designing the interchain connecting peptide and construction of single-chain antibody, but also a foundation for the targeted therapy of f liver cancer.

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Disclosure of conflict of interest

None.

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